Molecular Mechanisms of Lymphangiogenesis

T. MÄKINEN AND K. ALITALO
Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Haartman Institute and Helsinki University Hospital, University of Helsinki, 00014 Helsinki, Finland

Blood and lymphatic vessels together form a circulatory system, which allows the transportation of metabolic substances, cells, and proteins in the body. A major role of the lymphatic vasculature is to return an excess of the protein-rich interstitial fluid to the blood circulation. In addition, the lymphatic vasculature is an important part of the immune system, as it filters lymph and its antigens through the lymph nodes. Lymphatic vessels also serve as one of the major routes for absorption of lipids from the gut (Witte et al. 2001). Until recently, studies of the lymphatic vessels were hampered due to the lack of specific markers, but recently several such markers have been identified. In addition, recent studies have indicated an important role for the lymphatic vessels in certain developmental disorders, such as lymphedema, and as a route for the metastasis of malignant tumors. These findings have brought lymphatic vascular biology to the forefront of cardiovascular research.

Two members of the vascular endothelial growth factor (VEGF) family of growth factors, VEGF-C and VEGF-D, have been shown to stimulate the growth of lymphatic vessels via the lymphatic endothelial cell receptor, VEGFR-3 (Jeltsch et al. 1997; Veikkola et al. 2001). Although VEGFR-3 is required for the development of the blood vascular system during early embryonic development, later on its expression becomes restricted to lymphatic endothelial cells and it thus serves as a marker of these cells in adult tissues (Kaipainen et al. 1995; Dumont et al. 1998). Recent studies demonstrate that VEGFR-3 and its ligands are involved in the development of lymphedema and lymphatic metastasis (for review, see Karkkainen et al. 2001a; Karpanen and Alitalo 2001).

Studies in our laboratory have been targeted to the molecular mechanisms regulating the development and growth of the lymphatic vessels, concentrating on the role of the VEGFR-3 signal transduction pathway in these processes. We have shown that VEGFR-3 mediates signals for the proliferation, survival, and migration of lymphatic endothelial cells and demonstrated that VEGFR-3 function is required for the normal development of the lymphatic vasculature (Mäkinen et al. 2001a,b). Blocking of VEGFR-3 signaling inhibited specifically fetal lymphangiogenesis without affecting blood vessel development, indicating that distinct molecular mechanisms control the development of the two vascular systems. To find the basis for such a specificity in signaling, we have now extended our studies to the characterization of the genetic programs that determine the identities of blood vascular and lymphatic endothelial cells. Our studies give new insights into the phenotypic diversity of endothelial cells and reveal new potential vascular markers, some of which could provide important targets for the treatment of diseases characterized by abnormal angiogenesis or lymphangiogenesis.

DEVELOPMENT OF THE LYMPHATIC VASCULATURE

During embryogenesis, the cardiovascular system is the first functional organ system, and its development and growth are essential for the growth of the embryo. The endothelial cells lining the blood vessels differentiate from mesodermal precursor cells, angioblasts, to form a primitive vascular network in the process called vasculogenesis (Risau and Flamme 1995; Risau 1997). The primitive plexus of homogeneously sized endothelial tubes is then extensively remodeled into a more mature vessel network consisting of a hierarchy of larger and smaller blood vessels (Fig. 1). This remodeling occurs both by regression of some vessels and by angiogenesis, which consists of the sprouting, splitting, or fusion of preexisting vessels to adjust the vascular density according to the metabolic requirements of the respective tissue or organ (Risau 1997; Carmeliet 2000). Already before the onset of the blood circulation, the endothelial tubes are specified as two parallel but distinct vascular networks, the arteries and the veins. The normal sprouting and branching of the maturing vasculature is accompanied by repulsive signals that prevent arteries and veins from fusing together. Soon after the formation of the vessels, endothelial tubes become surrounded by pericytes or vascular smooth muscle cells (Fig. 1). Pericytes and endothelial cells share a common basement membrane (BM) containing fibronectin, laminin, collagens, and heparan sulfate proteoglycans. The composition of the vascular BM varies depending on the type of the vessel and on the tissue environment. Failure to form a proper BM can lead to decreased pericyte adhesion and migration and to defects in vessel stability, indicating an important role for the BM in normal vascular development (Beltramo et al. 2002; Thyboll et al. 2002).

The lymphatic vessels arise after the establishment of the blood circulatory system. In the early 20th century, Florence Sabin described the growth of lymphatic vessels in the pig fetus as centrifugal sprouting from the lymph sacs which are formed in the vicinity of certain fetal veins (Sabin 1902,1909). Recent molecular biological data support Sabin's theory of the venous origin of lymphatic ves-

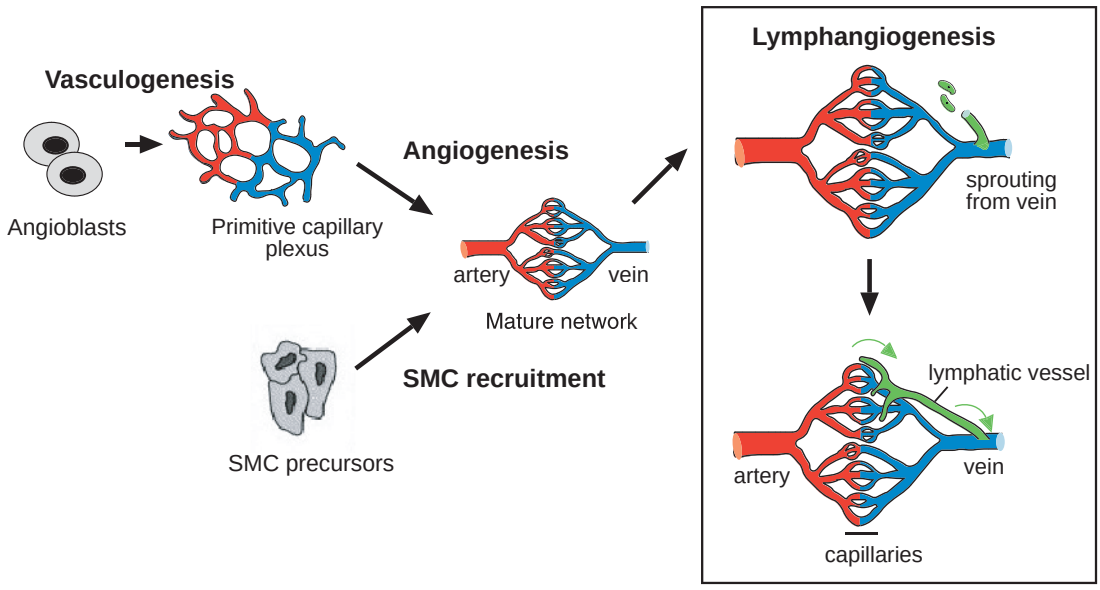

Figure 1. Development of the embryonic blood and lymphatic vessels. During vasculogenesis the endothelial precursor cells, angioblasts, form a primitive capillary plexus which is then further remodeled via angiogenesis. Vessel stabilization requires the recruitment of smooth muscle cells (SMC). Lymphatic vessels arise by sprouting from the veins, or they differentiate from mesodermal precursor cells. (Adapted, with permission, from Alitalo and Carmeliet 2002 [copyright Elsevier Science].)

sels (Dumont et al. 1998; Wigle and Oliver 1999; Wigle et al. 2002). A homeobox transcription factor Prox-1 has been found to have an important role in the formation of the lymph sacs by endothelial cell sprouting from the veins. At the stage when lymphatic development starts, Prox-1 is expressed in a subpopulation of endothelial cells of the anterior cardinal vein. These cells then migrate out and give rise to the lymphatic endothelial cells (Wigle and Oliver 1999). Targeted inactivation of Prox-1 leads to the arrest of the lymphatic endothelial budding process, whereas blood vessel development is not affected (Wigle and Oliver 1999).

Another old theory postulates that the lymphatic endothelial cells are derived from mesenchymal precursor cells (Huntington and McClure 1908; Kampmeier 1912). This theory is supported by the recent finding of these precursors, lymphangioblasts, in avian embryonic mesenchyme (Schneider et al. 1999; Papoutsi et al. 2001). Therefore, at least during embryogenesis, both the sprouting lymphangiogenesis and the mesenchymal cell differentiation into lymphatic endothelial cells may contribute to the formation of the lymphatic vessels (Fig. 1).

MOLECULAR REGULATION OF LYMPHANGIOGENESIS

VEGF, acting mainly via the VEGFR-1 and VEGFR-2 receptor tyrosine kinase receptors, is one of the most important regulators of both physiological and pathological angiogenesis (for review, see Ferrara 1999). In contrast, two other members of the VEGF family, VEGF-C and VEGF-D, have been shown to induce the growth of lymphatic vessels via the lymphatic endothelial cell receptor VEGFR-3 (Jeltsch et al. 1997; Veikkola et al. 2001). VEGF-C and VEGF-D are expressed as prepro-proteins which are proteolytically processed into polypeptides with increasing affinity toward VEGFR-3, and only their fully processed forms can bind to and activate VEGFR-2 (Joukov et al. 1997; Stacker et al. 1999). During embryonic development, VEGF-C is expressed in the mesenchyme surrounding the developing lymphatic vessels (Kukk et al. 1996). This suggested its possible role in the regulation of lymphangiogenesis. Subsequently, both VEGF-C and VEGF-D were shown to induce lymphatic growth in vivo (Jeltsch et al. 1997; Oh et al. 1997; Veikkola et al. 2001) and to induce the proliferation and migration of (lymphatic) endothelial cells in vitro (Marconcini et al. 1999; Mäkinen et al. 2001b). At higher concentrations and under specific conditions, these factors can also stimulate the proliferation and migration of blood vascular endothelial cells and increase vascular permeability and angiogenesis (Joukov et al. 1997; Oh et al. 1997; Witzenbichler et al. 1998; Mäkinen et al. 2001b). The VEGF-C/D-induced effects on blood vessel endothelium are presumably mediated via VEGFR-2 activation, suggesting an important role for specific proteolytic enzymes in regulating the lymphangiogenic versus angiogenic potentials of VEGF-C and VEGF-D.

VEGFR-3 is first expressed in developing blood vessels and is required for normal vascular development before midgestation. In the absence of VEGFR-3, the embryos undergo normal vasculogenesis, but the remodeling and maturation of primary vascular networks is defective, which leads to death in utero around E10 (Dumont et al. 1998). However, after the formation of the lymphatic vessels, VEGFR-3 is detected almost exclu-

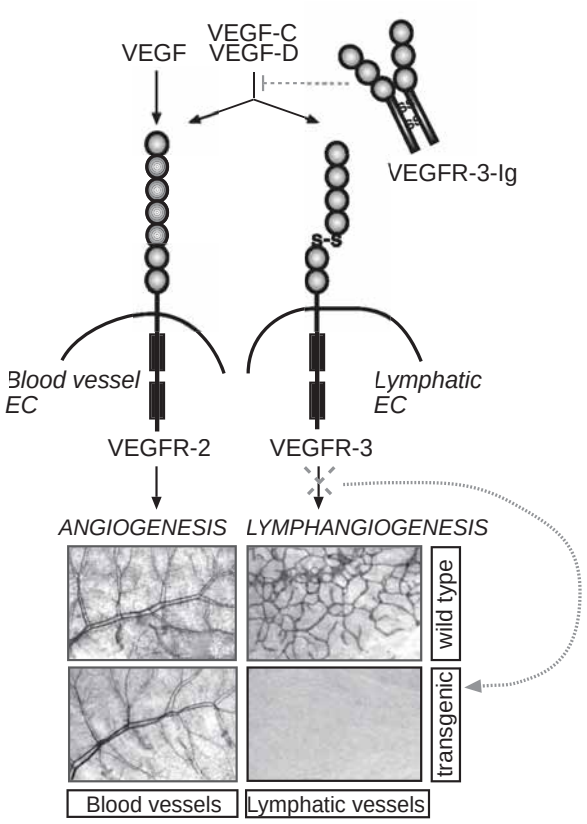

Figure 2. Inhibition of VEGFR-3 signaling by the transgenic expression of a soluble VEGFR-3-immunoglobulin (Ig)-fusion protein. Visualization of dermal blood and lymphatic vessels in wild-type and transgenic mice. Inhibition of VEGFR-3 signaling leads to a specific inhibition of lymphangiogenesis.

sively in the lymphatic endothelium (Kaipainen et al. 1995; Dumont et al. 1998). Because the *VEGFR-3*$^{-/-}$ embryos die before the emergence of the lymphatic vessels, these mice did not provide any information on the role of VEGFR-3 in the development of lymphatic vessels (Dumont et al. 1998). To address this question, we used a soluble VEGFR-3 as a competitive inhibitor of the VEGF-C/D - VEGFR-3 signaling (Fig. 2). Mice expressing a soluble VEGFR-3 immunoglobulin-fusion protein in the skin keratinocytes were found to be devoid of the dermal lymphatic vessels whereas the blood vasculature remained normal, showing a strikingly specific effect on the lymphatic endothelium (Mäkinen et al. 2001a). The early development of the lymphatic vasculature was not disturbed in the transgenic mice. However, in parallel with a significant increase in transgene-encoded protein expression, the dermal lymphatic vessels regressed in the transgenic embryos via apoptosis of lymphatic endothelial cells. Therefore, our results suggest that continuous VEGFR-3 signaling is required not only for the proliferation, but also for the survival, of the lymphatic endothelial cells and thus for both the development and maintenance of the lymphatic vasculature during embryonic development.

Neutralizing levels of circulating soluble VEGFR-3-Ig protein were also associated with a transient loss of lymphatic vessels in several internal organs of the neonatal transgenic mice. However, after three weeks of age, partial regeneration of the lymphatic vessels was observed, suggesting that alternative pathways may be involved in mediating the growth of the lymphatic vessels. For example, the maturing extracellular matrix may provide signals for the survival and proliferation of lymphatic endothelial cells, and new lymphatic vessels may grow alongside the existing blood vessels while receiving growth signals from them. Infection of newborn mouse pups with a VEGFR-3-Ig-encoding adenovirus resulted also in the regression of the lymphatic vessels, but the vessels regenerated in a few weeks despite the high serum levels of the VEGFR-3-Ig protein (T. Karpanen et al., unpubl.). However, in adult mice AdVEGFR-3-Ig failed to induce the regression of normal lymphatic vessels (Karpanen et al. 2001 and unpubl.; He et al. 2002). These results suggest that the lymphatic vessels undergo some type of maturation process, after which their growth and survival may no longer be dependent on VEGF-C/D. Pericyte recruitment is known to act as a signal for blood vessel maturation, after which the endothelial cells can survive in the absence of VEGF (Benjamin et al. 1998).

Recent studies indicate a significant interplay between the angiopoietins and VEGFs in the formation of the blood and lymphatic vessels. Low VEGF levels combined with high expression of Ang-2 lead to vascular regression, whereas Ang-1 and VEGF seem to cooperate in promoting angiogenesis. Interestingly, Ang-1 protects the vasculature against VEGF-induced plasma leakage (Thurston et al. 1999, 2000). On the other hand, Ang-2 may be involved in the regulation of lymphangiogenesis, since inactivation of the *Ang2* gene leads to nonfunctional and disconnected lymphatic vessels in mouse embryos (Gale et al. 2002). Cross-regulation also exists, as both VEGF and VEGF-C increase Ang-2 mRNA in endothelial cells (Mandriota and Pepper 1998; Oh et al. 1999; T. Veikkola et al., in prep.).

LYMPHATIC VESSELS IN TUMORS AND LYMPHATIC METASTASIS

Tumor metastasis by direct invasion or via blood or lymphatic vessels is one of the leading causes of death in cancer patients (Plate 2001). Although hematogenous metastasis via blood vessels is well recognized, for many tumors the most common pathway for metastatic spread is via the lymphatic vessels. In addition, the lymphatic spread of tumor cells to regional lymph nodes is an indicator of tumor aggressiveness. In general, it is thought that a high interstitial pressure of tumors prevents the growth of lymphatic vessels into the tumor cell mass (Carmeliet and Jain 2000). However, the studies of the involvement of the lymphatic vessels in tumor growth were hampered for a long time due to absence of specific markers of the lymphatic endothelium.

Several experimental studies have recently demonstrated an important role for lymphatic vessels as a route

for tumor metastasis. Overexpression of the VEGFR-3 ligands, VEGF-C or VEGF-D, in tumor cells induced lymphangiogenesis in the tumor periphery, and in some cases also intralymphatic tumor growth and metastasis were obtained. These studies were done using transfected cells (Karpanen et al. 2001; Skobe et al. 2001; Stacker et al. 2001) or transgenic mice (Mandriota et al. 2001), but correlation of VEGF-C expression with increased lymphatic metastasis has also been reported in several human cancers (Ohta et al. 1999; Tsurusaki et al. 1999; Yonemura et al. 1999). In addition, when highly metastatic human tumor cells were selected in mice by serial selection via the lymph nodes, the selected cells expressed enhanced levels of VEGF-C (He et al. 2002). These studies suggest that VEGF-C- or VEGF-D-secreting tumors can activate the lymphatic vessels, which may then facilitate metastasis of tumor cells. Interestingly, tumor lymphangiogenesis and metastasis were inhibited by blocking VEGF-C or VEGF-D signaling by neutralizing antibodies against VEGFR-3 or by soluble VEGFR-3 protein (Karpanen et al. 2001; Stacker et al. 2001; He et al. 2002). This suggests that inhibition of VEGFR-3 signaling may provide a novel strategy to inhibit metastasis via lymphatic vessels.

LYMPHEDEMA

Lymphedema is a disorder characterized by insufficiency of the lymphatic system, which leads to accumulation of a protein-rich fluid in the tissues and to a disfiguring and disabling swelling of the extremities. In the chronic condition the patients also suffer from tissue fibrosis, adipose degeneration, impaired wound healing, and susceptibility to infections (Witte et al. 2001). Noninherited secondary or aquired lymphedema develops when the lymphatic vessels are damaged, for example, by surgery or radiation therapy, or obstructed by filarial infection. Inherited primary lymphedema is usually due to hypoplasia or aplasia of the superficial or subcutaneous lympatic vessels.

In some families of hereditary lymphedema, heterozygous missense mutations were found in the *VEGFR-3* gene. All the lymphedema-associated mutations inactivated the tyrosine kinase domain and therefore led to insufficient VEGFR-3 signaling (Irrthum et al. 2000; Karkkainen et al. 2000). However, primary lymphedemas are a heterogeneous group of disorders that are sometimes associated with additional malformations in other organs. Linkage to other genes has been found, although in some cases none has yet been identified. Genetically modified mouse mutants have provided important tools for the studies of molecular mechanisms involved in lymphatic growth and in the development of lymphedema (Table 1). A mouse model for human primary lymphedema was obtained using N-ethyl-N-nitrosourea-induced chemical mutagenesis. As in human lymphedema, heterozygous mice carrying an inactivating missense mutation in the *VEGFR-3* gene had hypoplastic dermal lymphatic vessels (Karkkainen et al. 2001b). In addition, the mice had leaky intestinal lymphatics, which resulted in accumulation of intraperitoneal chylous fluid after suckling. Since the mice carrying only one functional *VEGFR-3* allele appeared normal (Dumont et al. 1998), whereas the tyrosine kinase inactivating mutation was responsible for lymphedema, the phenotype was considered to arise due to a "dominant-negative" effect by the kinase inactive receptor. Despite this, gene therapy using adenoviral VEGF-C induced lymphangiogenesis in the lymphedema mice, suggesting its therapeutic application also for the human disease (Karkkainen et al. 2001b). The absence of dermal lymphatic vessels in the K14-VEGFR-3-Ig mice was associated with fibrosis, increased fluid accumulation in the skin and subcutaneous tissue, and swelling of the feet (Mäkinen et al. 2001a). This phenotype thus resembles human lymphedema, and the data support the view that defective VEGFR-3 signaling can cause the disease.

We have evidence that *VEGFR-3* haploinsufficiency can cause a lymphedema-like phenotype in mice (T. Mäkinen and K. Alitalo, unpubl.). The dependence of the phenotype on the genetic background resembles the observed variability in the penetrance of human lymphedema and suggests that genes modulating, e.g., VEGFR-3, signaling pathways may significantly contribute to the development of the disease. Similar genetic background-dependent lymphatic phenotype, determined by accumulation of chylous ascites, has been previously observed in mice deficient of transcription factor Sox18 (Pennisi et al. 2000). In endothelial cells, Sox18 interacts with a MADS-family transcription factor MEF2C, which potentiates its transcriptional activity (Hosking et al. 2001). Interestingly, targeted mutagenesis of MEF2C leads to a similar phenotype as observed in the VEGFR-3-deficient mice. MEF2C-deficient embryos die at E9.5–10 due to defects in the remodeling of the vasculature and abnormal endocardiogenesis (Lin et al. 1998; Bi et al. 1999). Although the endocardial defects may be caused by a significant decrease in Ang-1 and VEGF expression in the myocardium (Bi et al. 1999), decreased VEGFR-3 expression may contribute to the failure in vascular remodeling. In line with this hypothesis, the VEGFR-3 promoter region contains a conserved binding motif for MEF2 (Iljin et al. 2001). Therefore, both Sox18 and MEF2C may participate in a common transcriptional program involved in the development of the lymphatic vasculature.

MOLECULAR IDENTITY OF LYMPHATIC VERSUS BLOOD VASCULAR ENDOTHELIAL CELLS

In addition to neovascularization, the endothelium has a variety of other important functions in, e.g., regulation of the vascular tone, maintenance of the blood-brain barrier, and inflammatory responses. For example, endothelial cells recruit leukocytes to inflammatory foci, and specialized endothelial cells are responsible for the homing of lymphocytes to the secondary lymphoid organs (Biedermann 2001). Reflecting such functional diversity, the

Table 1. Mouse Mutants Having a Lymphatic Phenotype

Gene	Mouse model[a]	Lymphatic phenotype	Lethality[b]	Reference
Prox-1	KO	defective lymphatic sprouting and differentiation (–/–)	E14.5	Wigle and Oliver (1999)
		accumulation of chylous ascites (+/–)	P2-3	
Integrin α9	KO	chylothorax (–/–)	P6-12	Huang et al. (2000)
Ang-2	KO	disconnected lymphatic vessels, accumulation of chylous ascites		N. Gale et al. (pers. comm.)
VEGFR-3	Mut (kinase dead)	accumulation of chylous ascites, hypoplasia of dermal lymphatic vessels (+/mut)	viable	Karkkainen et al. (2001b)
Net transcription factor	Mut (loss of the Ets DNA-binding domain)	chylothorax (mut/mut)	P2	Ayadi et al. (2001)
Sox18 transcription factor	Mut (truncated *trans*-activation domain)	accumulation of chylous ascites (mut/mut)	before weaning	Pennisi et al. (2000)
VEGF-C	TG (K14 promoter)	hyperplasia of dermal lymphatic vessels	viable	Jeltsch et al. (1997)
	(insulin promoter)	hyperplasia of lymphatic vessels around the islets of Langerhans	viable	Mandriota et al. (2001)
VEGF-D	TG (K14 promoter)	hyperplasia of dermal lymphatic vessels	viable	Veikkola et al. (2001)
VEGFR-3-Ig (VEGF-C/D "trap")	TG (K14 promoter)	lack of dermal lymphatic vessels	viable	Mäkinen et al. (2001a)

[a]KO = knock-out (gene inactivation), Mut = mutagenesis (chemically induced, VEGFR-3) / gene-targeted (Net) / spontaneous (Sox18), TG = transgenic (gene overexpression).
[b]E = embryonic day, P = postnatal day.

antigenic profile of the vascular endothelium varies in different types of vessels and in different organs.

Lymphatic marker identification has enabled detailed studies of the lymphatic vessels during the last few years. These lymphatic endothelium-specific molecules include VEGFR-3 receptor tyrosine kinase, the Prox-1 transcription factor, the hyaluronen receptor LYVE-1, and the membrane mucoprotein podoplanin (Table 2) (Jussila et al. 1998; Banerji et al. 1999; Breiteneder-Geleff et al. 1999; Wigle and Oliver 1999). None of these molecules is specific for endothelial cells and, on the other hand, they may be expressed only in a subset of the lymphatic vessels. In addition, the function of many of these molecules in lymphatic endothelial cell biology remains to be characterized.

Identification of differentially expressed genes between blood vascular and lymphatic endothelial cells may provide new potential vascular markers and also give a better understanding of the structural and functional properties of these cells. In our studies, we have found that the microvascular endothelial cell cultures consist of distinct lineages of blood vascular and lymphatic endothelial cells (Fig. 3). The lymphatic endothelial cells (LECs) grew in distinct islands surrounded by blood vascular endothelial cells (BECs). We used antibodies against VEGFR-3 or podoplanin and magnetic microbeads for the separation of BECs and LECs, which were then grown as separate cell cultures (Mäkinen et al. 2001b). No interconversion between the cell types was detected in these cultures. In an independent study by Kriehuber et al. (2001), the BECs and LECs were separated from dermal cell suspensions by flow cytometry using antibodies against podoplanin and CD34 (Kriehuber et al. 2001). In agreement with our results, their studies demonstrated that the LECs and BECs constitute stable and specialized EC lineages.

Table 2. Examples of Lymphatic Endothelial Cell Markers

Protein	Function	Reference
VEGFR-3	receptor tyrosine kinase; development of blood vasculature, proliferation, survival and migration of LECs	Jussila et al. (1998); Mäkinen et al. (2001a,b)
LYVE-1	lymphatic hyaluronan receptor	Banerji et al. (1999)
Prox-1	homeobox transcription factor; differentiation of LECs	Wigle and Oliver (1999)
Desmoplakin I and II	intercellular adherens junction molecule	Ebata et al. (2001)
Podoplanin	integral membrane protein	Breiteneder-Geleff et al. (1999)
D6	β-chemokine receptor	Nibbs et al. (2001)
Macrophage mannose receptor I	endocytotic/phagocytotic receptor in macrophages	Irjala et al. (2001)

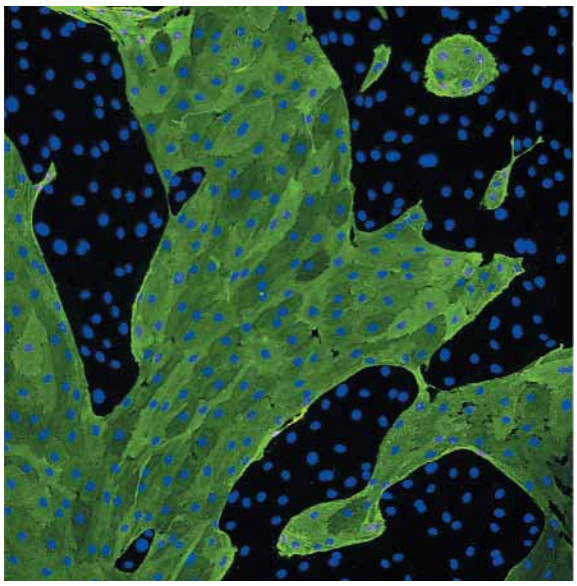

Figure 3. Human dermal microvascular endothelial cells in culture. The lymphatic endothelial cells (stained in green using antibodies against podoplanin) grow in distinct islands surrounded by blood vascular endothelial cells. The nuclei of all cells have been stained blue.

After establishing the isolation and culture of BECs and LECs, we have addressed the question of the genetic program controlling the identity of these two cell lineages using a gene profiling approach. We have identified so far about 300 genes that are differentially expressed between the cultured BECs and LECs (Petrova et al. 2002). The most striking differences were detected in genes that are involved in cytoskeletal and cell–cell or cell–matrix interactions, as well as in the expression of proinflammatory cytokines and chemokines.

Prox-1 AS A FATE-DETERMINING FACTOR FOR LYMPHATIC ENDOTHELIAL CELLS

Homeodomain transcription factors are important regulators of cellular fates and development. Gene-targeting studies have demonstrated that the Prox-1 homeobox transcription factor has a crucial role in the development of the lymphatic vasculature (Wigle and Oliver 1999). Prox-1 was found to be expressed specifically in the cultured LECs. However, when introduced into the BECs using adenoviral gene transfer, Prox-1 induced the expression of certain lymphatic-specific genes, including VEGFR-3 (Petrova et al. 2002). Interestingly, Prox-1 overexpression also resulted in the suppression of ~40% of the genes characteristic of the BECs. These results show that Prox-1 is able to reprogram gene expression in BECs toward the identity of LECs and suggest a role for Prox-1 as one of the fate-determining factors for the LECs.

Our findings and those reported by Wigle et al. (2002) suggest that in the absence of Prox-1 the default endothelial cell differentiation state corresponds to the blood endothelial phenotype and that Prox-1 is required for the establishment of lymphatic endothelial cell identity. These results help to explain the defective lymphatic differentiation and sprouting observed in the Prox-1 null mice (Wigle and Oliver 1999). The first lymphatic sprouts normally bud from the anterior cardinal vein at E10.5, but this budding is arrested in *Prox-1*$^{-/-}$ embryos at E11.5. In contrast to the wild-type embryos, in *Prox-1*$^{-/-}$ embryos the endothelial cells that bud off from the cardinal vein at E11.5–E12 do not express the lymphatic endothelial cell markers VEGFR-3, LYVE-1, or SLC. Instead, the mutant cells expressed blood vascular markers laminin and CD34, suggesting that these cells were not committed to the lymphatic endothelial cell lineage in the absence of Prox-1 (Wigle et al. 2002).

Gene targeting also revealed that Prox-1 haploinsufficiency is associated with the accumulation of lymphatic chylous fluid and neonatal death, suggesting that the level of Prox-1 is critical for proper lymphatic development (Wigle and Oliver 1999). We also found that Prox-1 upregulated the expression of genes associated with S-phase progression (Petrova et al. 2002), suggesting that after the differentiation of lymphatic endothelial cells, Prox-1 may also be essential for the maintenance of their proliferation during development.

CONCLUSIONS

Lymphatic vessels are essential for the maintenance of normal fluid balance and immune surveillance, but they also provide a pathway for metastasis in many types of cancers. The VEGFR-3 receptor tyrosine kinase is expressed in the lymphatic endothelium and its ligands;VEGF-C and VEGF-D induce lymphangiogenesis. VEGFR-3 mediates signals for the survival, migration, and proliferation of lymphatic endothelial cells, and these signals are essential for the normal development of the lymphatic vessels during embryonic development. Inhibition of VEGFR-3 signaling by using a soluble VEGFR-3 protein resulted in a specific and complete inhibition of lymphangiogenesis in a mouse model. Additionally, in tumor models the soluble VEGFR-3 specifically inhibited lymphangiogenesis and tumor spread via the lymphatic vessels. In cancers that show lymphogenous metastasis, the blocking of the VEGFR-3 signaling pathway may thus provide a useful strategy to inhibit metastasis.

Until now, only a few lymphatic endothelial cell lines have been available for molecular biological studies, and these cells were mainly derived from lymphatic tumors. The ability to isolate and maintain primary cultures of dermal blood vascular and lymphatic endothelial cells (BECs and LECs, respectively) now allows the characterization of the molecular properties of these cells. Using gene expression profiling, we have identified genes that distinguish these two cell lineages. We have also shown that the homeobox transcription factor Prox-1 is involved in the establishment of the molecular identity of the LECs. Molecular discrimination of the blood vascular and lymphatic vessels is essential in studies of diseases involving these vessels and in the targeted treatment of

such diseases. Thus the BEC- and LEC-specific molecules may provide new targets for the treatment of such diseases.

Further challenges in the lymphatic research include the identification of new molecular regulators of lymphangiogenesis. The ability to isolate and to culture LECs facilitates the cloning of novel LEC-specific genes, the characterization of which should give us a better understanding of the mechanisms of lymphangiogenesis. The functional analysis of the new lymphangiogenic regulators may also give us novel insights into the pathogenesis of diseases of the lymphatic system.

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REFERENCES

Alitalo K. and Carmeliet P. 2002. Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* **1:** 219.

Ayadi A., Zheng H., Sobieszczuk P., Buchwalter G., Moerman P.,m alitalo K., and Wasylyk B. 2001 Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. *EMBO J.* **20:** 5139.

Banerji S., Ni J., Wang S.X., Clasper S., Su J., Tammi R., Jones M., and Jackson D.G. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol.* **144:** 789.

Beltramo E., Pomero F., Allione A., D'Alu F., Ponte E., and Porta M. 2002. Pericyte adhesion is impaired on extracellular matrix produced by endothelial cells in high hexose concentrations. *Diabetologia* **45:** 416.

Benjamin L.E., Hemo I., and Keshet E. 1998. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125:** 1591.

Bi W., Drake C.J., and Schwarz J.J. 1999. The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiopoietin 1 and VEGF. *Dev. Biol.* **211:** 255.

Biedermann B.C. 2001. Vascular endothelium: Checkpoint for inflammation and immunity. *News Physiol. Sci.* **16:** 84.

Breiteneder-Geleff S., Soleiman A., Kowalski H., Horvat R., Amann G., Kriehuber E., Diem K., Weninger W., Tschachler E., Alitalo K., and Kerjaschki D. 1999. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: Podoplanin as a specific marker for lymphatic endothelium. *Am. J. Pathol.* **154:** 385.

Carmeliet P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6:** 389.

Carmeliet P. and Jain R.K. 2000. Angiogenesis in cancer and other diseases. *Nature* **407:** 249.

Dumont D.J., Jussila L., Taipale J., Lymboussaki A., Mustonen T., Pajusola K., Breitman M., and Alitalo K. 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* **282:** 946.

Ebata N., Nodasaka Y., Sawa Y., Yamaoka Y., Makino S., Totsuka Y., and Yoshida S. 2001. Desmoplakin as a specific marker of lymphatic vessels. *Microvasc. Res.* **61:** 40-48.

Ferrara N. 1999. Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* **77:** 527.

Gale N.W., Thurston G., Hackett S.F., Renard R., Wang Q., McClain J., Martin C., Witte C., Witte M.H., Jackson D., Suri C., Campochiaro P.A., Wiegand S.J., and Yancopoulos G.D. 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev. Cell* **3:** 411.

He Y., Kozaki K., Karpanen T., Koshikawa K., Yla-Herttuala S., Takahashi T., and Alitalo K. 2002. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J. Natl. Cancer Inst.* **5:** 819.

Hosking B.M., Wang S.C., Chen S.L., Penning S., Koopman P., and Muscat G.E. 2001. SOX18 directly interacts with MEF2C in endothelial cells. *Biochem. Biophys. Res. Commun.* **287:** 493.

Huang X.Z., Wu J.F., Ferrando R., Lee J.H., Wang Y.L, Farese R.V., Jr., and Sheppard D. 2000. Fatal bilateral chylothorax in mice lacking the integrin α9β1. *Mol. Cell. Biol.* **20:** 5208.

Huntington G.S. and McClure C.F.W. 1908. The anatomy and development of the jugular lymph sac in the domestic cat (*Felis domestica*). *Anat. Rec.* **2:** 1.

Iljin K., Karkkainen M.J., Lawrence E.C., Kimak M.A., Uutela M., Taipale J., Pajusola K., Alhonen L., Halmekyto M., Finegold D.N., Ferrell R.E., and Alitalo K. 2001. VEGFR3 gene structure, regulatory region, and sequence polymorphisms. *FASEB J.* **15:** 1028.

Irjala H., Johansson E.L., Grenman R., Alanen K., Salmi M., and Jalkanen S. 2001. Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. *J. Exp. Med.* **194:** 1033.

Irrthum A., Karkkainen M.J., Devriendt K., Alitalo K., and Vikkula M. 2000. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am. J. Hum. Genet.* **67:** 295.

Jeltsch M., Kaipainen A., Joukov V., Meng X., Lakso M., Rauvala H., Swartz M., Fukumura D., Jain R.K., and Alitalo K. 1997. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276:** 1423.

Joukov V., Sorsa T., Kumar V., Jeltsch M., Claesson-Welsh L., Cao Y., Saksela O., Kalkkinen N., and Alitalo K. 1997. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J.* **16:** 3898.

Jussila L., Valtola R., Partanen T.A., Salven P., Heikkila P., Matikainen M.T., Renkonen R., Kaipainen A., Detmar M., Tschachler E., Alitalo R., and Alitalo K. 1998. Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res.* **58:** 1599.

Kaipainen A., Korhonen J., Mustonen T., van Hinsbergh V.M., Fang G.-H., Dumont D., Breitman M. and Alitalo K. 1995. Expression of the *fms*-like tyrosine kinase FLT4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci.* **92:** 3566.

Kampmeier O.F. 1912. The value of the injection method in the study of lymphatic development. *Anat. Rec.* **6:** 223.

Karkkainen M.J., Jussila L., Ferrell R.E., Finegold D.N., and Alitalo K. 2001a. Molecular regulation of lymphangiogenesis and targets for tissue oedema. *Trends Mol. Med.* **7:** 18.

Karkkainen M.J., Ferrell R.E., Lawrence E.C., Kimak M.A., Levinson K.L., Alitalo K., and Finegold D.N. 2000. Missense mutations interfere with VEGFR-3 signaling in primary lymphoedema. *Nat. Genet.* **25:** 153.

Karkkainen M.J., Saaristo A., Jussila L., Karila K.A., Lawrence E.C., Pajusola K., Bueler H., Eichmann A., Kauppinen R., Kettunen M.I., Yla-Herttuala S., Finegold D.N., Ferrell R.E., and Alitalo K. 2001b. A model for gene therapy of human hereditary lymphedema. *Proc. Natl. Acad. Sci.* **98:** 12677.

Karpanen T. and Alitalo K. 2001. Lymphatic vessels as targets of tumor therapy? *J. Exp. Med.* **194:** F37.

Karpanen T., Egeblad M., Karkkainen M.J., Kubo H., Ylä-Herttuala S., Jäättelä M., and Alitalo K. 2001. Vacular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res.* **61:** 1786.

Kriehuber E., Breiteneder-Geleff S., Groeger M., Soleiman A., Schoppmann S.F., Stingl G., Kerjaschki D., Maurer D. 2001.

Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J. Exp. Med.* **194:** 797.

Kukk E., Lymboussaki A., Taira S., Kaipainen A., Jeltsch M., Joukov V., and Alitalo K. 1996. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggest a role in lymphatic vascular development. *Development* **122:** 3829.

Lin Q., Lu J., Yanagisawa H., Webb R., Lyons G.E., Richardson J.A., and Olson E.N. 1998. Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development* **125:** 4565.

Mäkinen T., Jussila L., Veikkola T., Karpanen T., Kettunen M.I., Pulkkanen K.J., Kauppinen R., Jackson D.G., Kubo H., Nishikawa S.-I., Ylä-Herttuala S., and Alitalo K. 2001a. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat. Med.* **7:** 199.

Mäkinen T., Veikkola T., Mustjoki S., Karpanen T., Wise L., Mercer A., Catimel B., Nice E.C., Kowalski H., Kerjaschki D., Stacker S.A., Achen M.G., and Alitalo K. 2001b. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J.* **20:** 4762.

Mandriota S.J. and Pepper M.S. 1998. Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ. Res.* **83:** 852.

Mandriota S.J., Jussila L., Jeltsch M., Compagni A., Baetens D., Prevo R., Banerji S., Huarte J., Montesano R., Jackson D.G., Orci L., Alitalo K., Christofori G., and Pepper M.S. 2001. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* **20:** 672.

Marconcini L., Marchio S., Morbidelli L., Cartocci E., Albini A., Ziche M., Bussolino F., and Oliviero S. 1999. c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. *Proc. Natl. Acad. Sci.* **96:** 9671.

Nibbs R.J., Kriehuber E., Ponath P.D., Parent D., Qin S., Campbell J.D., Henderson A., Kerjaschki D., Maurer D., Graham G.J., and Rot A. 2001. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am. J. Pathol.* **158:** 867.

Oh H., Takagi H., Suzuma K., Otani A., Matsumura M., and Honda Y. 1999. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J. Biol. Chem.* **274:** 15732.

Oh S.J., Jeltsch M.M., Birkenhager R., McCarthy J.E., Weich H.A., Christ B., Alitalo K., and Wilting J. 1997. VEGF and VEGF-C: Specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev. Biol.* **188:** 96.

Ohta Y., Shridhar V., Bright R.K., Kalemkerian G.P., Du W., Carbone M., Watanabe Y., and Pass H.I. 1999. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br. J. Cancer* **81:** 54.

Papoutsi M., Tomarev S.I., Eichmann A., Prols F., Christ B., and Wilting J. 2001. Endogenous origin of the lymphatics in the avian chorioallantoic membrane. *Dev. Dyn.* **222:** 238.

Pennisi D., Gardner J., Chambers D., Hosking B., Peters J., Muscat G., Abbott C., and Koopman P. 2000. Mutations in Sox18 underlie cardiovascular and hair follicle defects in ragged mice. *Nat. Genet.* **24:** 434.

Petrova T.V., Mäkinen T., Mäkelä T.P., Saarela J., Virtanen I., Ferrell R.E., Finegold D.N., Kerjaschki D., Ylä-Herttuala S., and Alitalo K. 2002. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 home box transcription factor. *EMBO J.* **21:** 4593.

Plate K.H. 2001. From angiogenesis to lymphangiogenesis. *Nat. Med.* **7:** 151.

Risau W. 1997. Mechanisms of angiogenesis. *Nature* **386:** 671.

Risau W. and Flamme I. 1995. Vasculogenesis. *Annu. Rev. Cell Dev. Biol*. **11:** 73.

Sabin F.R. 1902. On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. *Am. J. Anat.* **1:** 367.

———. 1909. The lymphatic system in human embryos, with a consideration of the morphology of the system as a whole. *Am. J. Anat.* **9:** 43.

Schneider M., Othman-Hassan K., Christ B., and Wilting J. 1999. Lymphangioblasts in the avian wing bud. *Dev. Dyn.* **216:** 311.

Skobe M., Hawighoest T., Jackson D.G., Prevo R., Janes L., Velasco P., Riccardi L., Alitalo K., Claffey K., and Detmar M. 2001. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* **7:** 192.

Stacker S.A., Caesar C., Baldwin M.E., Thornton G.E., Williams R.A., Prevo R., Jackson D.G., Nishikawa S.-I., Kubo H., and Achen M.G. 2001. Vascular endothelial growth factor-D promotes the metastatic spread of cancer via the lymphatics. *Nat. Med.* **7:** 186.

Stacker S.A., Stenvers K., Caesar C., Vitali A., Domagala T., Nice E., Roufail S., Simpson R.J., Moritz R., Karpanen T., Alitalo K., and Achen M.G. 1999. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J. Biol. Chem.* **274:** 32127.

Thurston G., Suri C., Smith K., McClain J., Sato T.N., Yancopoulos G.D., and McDonald D.M. 1999. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286:** 2511.

Thurston G., Rudge J.S., Ioffe E., Zhou H., Ross L., Croll S.D., Glazer N., Holash J., McDonald K., and Yancopoulos G.D. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat. Med.* **6:** 460.

Thyboll J., Kortesmaa J., Cao R., Soininen R., Wang L., Iivanainen A., Sorokin L., Risling M., Cao Y., and Tryggvason K. 2002. Deletion of the laminin alpha4 chain leads to impaired microvessel maturation. *Mol. Cell. Biol.* **22:** 1194.

Tsurusaki T., Kanda S., Sakai H., Kanetake H., Saito Y., Alitalo K., and Koji T. 1999. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br. J. Cancer* **80:** 309.

Veikkola T., Jussila L., Makinen T., Karpanen T., Jeltsch M., Petrova T.V., Kubo H., Thurston G., McDonald D.M., Achen M.G., Stacker S.A., and Alitalo K. 2001. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* **20:** 1223.

Wigle J.T. and Oliver G. 1999. Prox1 function is required for the development of the murine lymphatic system. *Cell* **98:** 769.

Wigle J.T., Harvey N., Detmar M., Lagutina I., Grosveld G., Gunn M.D., Jackson D.G., and Oliver G. 2002. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* **21:** 1505.

Witte M.H., Bernas M.J., Martin C.P., and Witte C.L. 2001. Lymphangiogenesis and lymphangiodysplasia: From molecular to clinical lymphology. *Microsc. Res. Tech.* **55:** 122.

Witzenbichler B., Asahara T., Murohara T., Silver M., Spyridopoulos I., Magner M., Principe N., Kearney M., Hu J.S., and Isner J.M. 1998. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am. J. Pathol.* **153:** 381.

Yonemura Y., Endo Y., Fujita H., Fushida S., Ninomiya I., Bandou E., Taniguchi K., Miwa K., Ohoyama S., Sugiyama K., and Sasaki T. 1999. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin. Cancer Res.* **5:** 1823.

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